



002.00041

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Cohen et al.)
Serial No.: 09/845,667) Examiner:
Filed: April 30, 2001) Art Unit: 1653
For: CONTROL OF PROTEIN SYNTHESIS)
AND SCREENING METHOD FOR)
AGENTS)

AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This Amendment is filed in response to the May 25, 2001 Notice to File Missing Parts issued in connection with the above identified application. A response to the Notice was originally due July 25, 2001. However, this Amendment is accompanied by a Petition for Extension of Time (4 months) and therefore a response is now due November 25, 2001.

Please amend the subject application as follows:

In the Specification:

Pursuant to 37 C.F.R. §1.121(b)(iii), a marked-up copy of the following amended paragraphs on a separate sheet accompanies this amendment.

Replace the paragraph on page 4, lines 8-19, with the following new paragraph:

The present invention also provides a novel peptide comprising the amino acid sequence Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd (SEQ ID NO:4), where Xaa is any amino acid, Yaa and Zaa are any amino acid (preferably not glycine), and Hyd is a large hydrophobic residue such as Phe or Leu, or a functional equivalent thereof. Represented in single letter code, a suitable peptide would be RXXR'X'S/TF/L (SEQ ID NO:4), where X' can be any amino acid, but is preferably not glycine; glycine can in fact

be used, but other amino acids are preferred. Typical peptides include GRPRTSSFAEG (SEQ ID NO:1), RPRAATC (SEQ ID NO:2) or functional equivalents thereof. The peptide is a substrate for measuring PKB activity.

Replace the paragraph on page 11, lines 22-35, with the following new paragraph:

Mono Q chromatography was performed as described (Burgering et al., 1995), except that the buffer also contained 1 mM EGTA, 0.1 mM sodium orthovanadate and 0.5% (w/v) Triton X-100. Two PKB α antibodies were raised in rabbits against the C-terminal peptide FPQFSYSASSTA (SEQ ID NO:3) and bacterially expressed PH domain of PKB α . The C-terminal antibody was affinity purified (Jones et al., 1991). The activity of PKB towards Crosstide is threefold higher than its activity towards histone H2B and 11-fold higher than its activity towards myelin basic protein, the substrates used previously to assay PKB. Other experimental details and units of protein kinase activity are given in Fig 1.

Replace the paragraph on page 15, lines 5-22, with the following new paragraph:

To identify the insulin-stimulated protein kinase that inhibits GSK3 in the presence of rapamycin and PD 98059, L6 myotubes were incubated with both compounds and stimulated with insulin. The lysates were then chromatographed on Mono Q and the fractions assayed with "Crosstide" (GRPRTSSFAEG) (SEQ ID NO:1), a peptide corresponding to the sequence in GSK3 surrounding the serine (underlined) phosphorylated by MAPKAP kinase-1 and p70^{S6k} (Ser 21 in GSK3- α (Sutherland et al., 1994) and Ser 9 in GSK3- β (Sutherland et al., 1993)). Three peaks of Crosstide kinase activity were detected, which were absent if insulin stimulation was omitted or if the cells were first preincubated with the PI 3-kinase inhibitor wortmannin (Fig 2a). Wortmannin (Cross et al., 1994 and

Welsh et al., 1994), and the structurally unrelated PI 3-kinase inhibitor LY 294002 (ref 19); (Fig 1b), both prevent the inhibition of GSK3 by insulin.

Replace the paragraph on page 25, lines 11-34, with the following new paragraph:

Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of PKB α causing partial activation. Ser-473 of PKB α lies in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr (SEQ ID NO:11) found to be conserved in a number of protein kinases that participate in signal transduction pathways (Pearson et al. 1995). In order to identify the Ser-473 kinase(s) we therefore chromatographed rabbit skeletal muscle extracts on CM-Sephadex, and assayed the fractions for protein kinases capable of phosphorylating a synthetic peptide corresponding to residues 465 to 478 of PKB α . These studies identified an enzyme eluting at 0.3 M NaCl which phosphorylated the peptide 465-478 at the residue equivalent to Ser-473 of PKB α . The Ser473 kinase co-eluted from CM-Sephadex with MAP kinase-activated protein (MAPKAP) kinase-2, (Stokoe et al., 1992) which is a component of a stress and cytokine-activated MAP kinase cascade (Rouse et al., 1994; Cuenda et al., 1995). The Ser-473 kinase continued to cofractionate with MAPKAP kinase-2 through phenyl-Sepharose, heparin-Sepharose, Mono S and Mono Q and was immunoprecipitated quantitatively by an anti-MAPKAP kinase-2 antibody (Gould et al., 1995) demonstrating that MAPKAP kinase-2 was indeed the Ser-473 kinase we had purified.

Replace the paragraph on page 33, line 23 through page 34, line 8, with the following new paragraph:

Thr-308 is located in subdomain VIII of the kinase catalytic domain, nine residues upstream of the conserved Ala-Pro-Glu motif, the same position as activating phosphorylation sites found in many other protein kinases. However, Ser-473 is located C-terminal to the

catalytic domain in the consensus sequence Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr (SEQ ID NO:11) which is present in several protein kinases that participate in growth factor-stimulated kinase cascades, such as p70 S6 kinase, PKC and p90rsk (Pearson et al., 1995). However, it is unlikely that a common protein kinase phosphorylates this motif in every enzyme for the following reasons.

Firstly, phosphorylation of the equivalent site in p70 S6 kinase is prevented by the immunosuppressant drug rapamycin (Pearson et al., 1995) which does not prevent the activation of PKB α by insulin (Cross et al., 1995) or its phosphorylation at Ser-473 (D. Alessi, unpublished work). Secondly, the equivalent residue in protein kinase C is phosphorylated constitutively and not triggered by stimulation with growth factors (Tsutakawa et al., 1995).

Replace the paragraph on page 34, line 10 through page 35, line 9, with the following new paragraph:

MAPKAP kinase-2 is a component of a protein kinase cascade which becomes activated when cells are stimulated with interleukin-1 or tumour necrosis factor or exposed cellular stresses (Rouse et al., 1994; Cuenda et al., 1995). MAPKAP kinase-2 phosphorylates PKB α stoichiometrically at Ser-473 (Fig 11) and this finding was useful in establishing the role of Ser473 phosphorylation in regulating PKB α activity. However, although MAPKAP kinase-2 activity is stimulated to a small extent by insulin in L6 cells, no activation could be detected in 293 cells in response to insulin or IGF-1. Moreover, exposure of L6 cells or 293 cells to a chemical stress (0.5 mM sodium arsenite) strongly activated MAPKAP kinase-2 (D. Alessi, unpublished work) as found in other cells (Rouse et al., 1994; Cuenda et al., 1995), but did not activate PKB α at all. Furthermore, the drug SB 203580 which is a specific inhibitor of the protein

kinase positioned immediately upstream of MAPKAP kinase-2 (Cuenda et al., 1995), prevented the activation of MAPKAP kinase-2 by arsenite but had no effect on the activation of PKB α by insulin or IGF-1. Finally, the activation of PKB α was prevented by wortmannin (Figs 6 and 9), but wortmannin had no effect on the activation of MAPKAP kinase-2 in L6 or 293 cells. It should also be noted that the sequence surrounding Ser-473 of PKB α (HFPQFSY) (SEQ ID NO:12) does not conform to the optical consensus for phosphorylation by MAPKAP kinase-2 which requires Arg at position n-3 and a bulky hydrophobic residue at position n-5, (where n is the position of the phosphorylated residue). The K_m for phosphorylation of the synthetic peptide comprising residues 465-478 is nearly 100-fold higher than the K_m for the standard MAPKAP kinase-2 substrate peptide (data not shown). It is therefore unlikely that MAPKAP kinase-2 mediates the phosphorylation of Ser-473 in vivo.

Replace the paragraph on page 37, lines 10-31, with the following new paragraph:

The activation of PI 3-kinase is essential for many of the effects of insulin and growth factors, including the stimulation of glucose transport, fatty acid synthesis and DNA synthesis, protection of cells against apoptosis and actin cytoskeletal rearrangements (reviewed in Carpenter et al., 1996). These observations raise the question of whether PKB α mediates any of these events by phosphorylating other proteins. To address this issue we characterised the substrate specificity requirements of PKB α . We find that the optimal consensus sequence for phosphorylation by PKB α is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd (SEQ ID NO:4), where Yaa and Zaa are small amino acids (other than glycine) and Hyd is a large hydrophobic residue (such as Phe or Leu). We also demonstrate that PKB α phosphorylates histone H2B (a

substrate frequently used to assay PKB α in vitro) at Ser-36 which lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (SEQ ID NO:4). These studies identified a further PKB α substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe) (SEQ ID NO:6) that, unlike other peptides, is not phosphorylated to a significant extent by either p70 S6 kinase or MAPKAP-K1. Replace the paragraph on page 38, line 23 through page 39, line 11, with the following new paragraph:

Identification of the residues in histone H2B

phosphorylated by PKB α . Currently, three substrates are used to assay PKB α activity in different laboratories, histone H2B, MBP and Crosstide. PKB α phosphorylated Crosstide with a Km of 4 μ M and a Vmax of 260 U/mg (Table 7.1 A, peptide 1), histone H2B with a Km of 5 μ M and a Vmax of 68 U/mg, and MBP with a Km of 5 μ M and a Vmax of 25 U/mg. Thus the Vmax of histone H2B and MBP are 4-fold and 10-fold lower than for Crosstide. In order to identify the residue(s) in histone H2B phosphorylated by PKB α , 32 P-labelled histone H2B was digested with trypsin (see Methods) and the resulting peptides chromatographed on a C18 column at pH 1.9. Only one major 32 P-labelled peptide (termed T1) eluting at 19.5 % acetonitrile was observed (Fig 17A). The peptide contained phosphoserine (data not shown), its sequence commenced at residue 34 of histone H2B and a single burst of radioactivity occurred after the third cycle of Edman degradation (Fig 17B), demonstrating that PKB α phosphorylates histone H2B at Ser-36 within the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr (SEQ ID NO:7). Thus, like the serine phosphorylated in Crosstide, Ser-36 of histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (SEQ ID NO:5) (where Hyd is a bulky hydrophobic residue, -Phe in Crosstide, Tyr in H2B).

Replace the paragraph on page 39, lines 13-32, with the following new paragraph:

Molecular basis for the substrate specificity of PKB α .

To further characterise the substrate specificity requirements for PKB α , we first determined the minimum sequence phosphorylated efficiently by PKB α by removing residues sequentially from the C-terminal and N-terminal end of Crosstide. Removal of the N-terminal glycine and up to three residues from the C-terminus had little effect on the kinetics of phosphorylation by PKB α (Table 7.1A, compare peptides 1 and 5). However any further truncation of either the N or C-terminus virtually abolished phosphorylation (Table 7.1A, peptides 8 and 9). The minimum peptide phosphorylated efficiently by PKB α (Arg-Pro-Arg-Thr-Ser-Ser-Phe) (SEQ ID NO:9) was found to be phosphorylated exclusively at the second serine residue as expected. Consistent with this finding, a peptide in which this serine was changed to alanine was not phosphorylated by PKB α (Table 7.1A, peptide 7). All further studies were therefore carried out using variants of peptide 5 in Table 7.1A (see below).

Replace the paragraph on page 40, line 29 through page 41, line 8, with the following new paragraph:

Replacement of the Thr situated two residues N-terminal to the phosphorylated serine increased the Km with any amino acid tested (Table 7.1C). Substitution with Ala only increased Km by 2-3 fold, but substitution with other residues was more deleterious and with Asn (a residue of similar size and hydrophilicity to Thr) phosphorylation was almost abolished (Table 7.1C). Replacement of the Ser situated one residue N-terminal to the phosphorylated serine also increased the Km with any amino acid tested, but the effects were less severe than at position n-2 (Table 7.1C). When residues n-2 and n-1 were both changed to Ala, the resulting peptide RPRAASF (SEQ ID NO:13) was phosphorylated by PKB α with a Km only 5-fold higher than RPRTSSF (SEQ ID NO:9). In contrast

the peptides RPRGGSF (SEQ ID NO:15), RPRAGSF (SEQ ID NO:16), and RPRGASF (SEQ ID NO:17) were phosphorylated less efficiently (Table 7.1C).

Replace the paragraph on page 41, lines 21-29, with the following new paragraph:

MAPKAP kinase-1 and p70 S6 kinase phosphorylate the peptides KKKNRTLVA (SEQ ID NO:18) and KKRNRTLVA (SEQ ID NO:19) with extremely low Km values of 0.2- 3.3 μ M, respectively (Table 7.2). However, these peptides were phosphorylated by PKB α with 50-900 fold higher Km values (Table 7.2A, peptides 1 and 2). The peptide KKRNRTLTV (SEQ ID NO:20), which is a relatively specific substrate for p70 S6 kinase (Leighton et al., 1995) was also phosphorylated very poorly by PKB α (Table 7.2A, peptide 4).

Replace the paragraph on page 41, line 31 through page 42, line 23, with the following new paragraph:

Crosstide is phosphorylated by p70 S6 kinase and MAPKAP kinase-1 with similar efficiency to PKB α ((Leighton et al., 1995); Table 7.2B-peptide 1 and Fig 18). However, truncation of Crosstide to generate the peptide RPRTSSF (SEQ ID NO:9) was deleterious for phosphorylation by MAPKAP-K1 and even worse for p70 S6 kinase (Table 7.2B-peptides 1 and 2 and Fig 18). Moreover, changing the phosphorylated serine in RPRTSSF (SEQ ID NO:9) to threonine increased the Km for phosphorylation by p70 S6 kinase much more than for PKB α and almost abolished phosphorylation by MAPKAP-K1 (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF (SEQ ID NO:13) was phosphorylated by MAPKAP-K1 with essentially identical kinetics to that of PKB α ; however phosphorylation by p70 S6 kinase was virtually abolished (Table 7.2B-peptide 4 and Fig 18). Based on these observations we synthesized the peptide RPRAATF (SEQ ID NO:21). This peptide was phosphorylated by PKB α with a Km of 25 μ M and similar Vmax

to RPRTSSF (SEQ ID NO:9), but was not phosphorylated to a significant extent by either MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig 18). In Fig 18, the protein kinase concentration in the assays towards Crosstide were 0.2 U/ml, and each peptide substrate was assayed at a concentration of 30 μ M. Filled bars denote PKB α activity, hatched bars MAPKAP kinase-1 activity, and grey bars p70 S6 kinase activity. The activities of each protein kinase are given relative to their activity towards Crosstide (100). The results are shown \pm SEM for two experiments each carried out in triplicate.

Replace the paragraph on page 42, line 25 through page 43, line 3, with the following new paragraph:

Discussion.

We have established that the minimum consensus sequence for efficient phosphorylation by PKB α is Arg-Xaa-Arg-Yaa-Zaa-Ser-Hyd (SEQ ID NO:10), where Xaa is any amino acid, Yaa and Zaa are small amino acids other than glycine (Ser, Thr, Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu) (Table 7.1). The heptapeptide with the lowest Km value was RPRTSSF (SEQ ID NO:9), its Km of 5 μ M being comparable to many of the best peptide substrates identified for other protein kinases. The Vmax for this peptide (250 nmoles min⁻¹ mg⁻¹) may be an underestimate because the PKB α was obtained by immunoprecipitation from extracts of IGF-1 stimulated 293 cells over-expressing this protein kinase, and a significant proportion of the PKB α may not have been activated by IGF-1 treatment.

Replace the paragraph on page 43, lines 5-20, with the following new paragraph:

The requirement for arginine residues at positions n-3 and n-5 (where n is the site of phosphorylation) seems important, because substituting either residue with lysine decreases phosphorylation drastically. Serine and threonine residues were preferred at positions n-1 and n-

2, although the K_m value was only increased about 5-fold if both of these residues were changed to Ala. Serine was preferred at position n, since changing it to threonine caused a six-fold increase in the K_m . The peptide RPRAATF (SEQ ID NO:21), which was phosphorylated with a K_m of 25 μ M and similar V_{max} to RPRTSSF (SEQ ID NO:9), may therefore be a better substrate for assaying PKB α in partially purified preparations, because unlike Crosstide, it contains only one phosphorylatable residue and is not phosphorylated significantly by MAPKAP-K1 or p70 S6 kinase (Table 7.2, Fig 18 and see below).

Replace the paragraph on page 43, lines 22-35, with the following new paragraph:

The Proline at position n-4 was not altered in this study because it was already clear that this residue was not critical for the specificity of PKB α . Residue n-4 is proline in GSK3 β but alanine in GSK3 α . Both GSK3 isoforms are equally good substrates for PKB α in vitro (Cross et al., 1995), and the peptide GRARTSSFA (SEQ ID NO:22) (corresponding to the sequence in GSK3 α) is phosphorylated by PKB α with a K_m of 10 μ M and V_{max} of 230 U/mg (Table 7.1A, peptide 2). Moreover, in histone H2B, the residue located four amino acids N-terminal to the PKB α phosphorylation site is serine (Fig 17). The presence of Glu and Lys at positions n-1 and n-2 may explain why histone H2B is phosphorylated by PKB α with a four-fold lower V_{max} than the peptide RPRTSSF (SEQ ID NO:9).

Replace the paragraph on page 44, line 22 through page 45, line 17, with the following new paragraph:

Additional similarities between p70 S6 kinase, MAPKAP-K1 and PKB α include the failure to phosphorylate peptides containing Pro at position n+1 and dislike of a lysine at the same position. This suggests that, in vivo, these kinases are unlikely to phosphorylate the same residues

as MAP kinases (which phosphorylate Ser/Thr-Pro motifs) or protein kinase C (which prefers basic residues C-terminal to the site of phosphorylation). However, the present work has also revealed significant differences in the specificities of these enzymes. In particular MAPKAP-K1 and (to a lesser extent) p70 S6 kinase can tolerate substitution of the Arg at position n-5 by lysine whereas PKB α cannot (see Table 7.1A, Table 7.2A and (Leighton et al., 1995)). MAPKAP-K1 and p70 S6 kinase can also tolerate, to some extent, substitution of Arg at position n-3 by Lys. For example, the peptide KKRNKTL SVA (SEQ ID NO:23) is phosphorylated by MAPKAP-K1 and p70 S6 kinase with Km values of 17 and 34 μ M, respectively, as compared to Km values of 0.7 and 1.5 μ M for the peptide KKRNR T L SVA (SEQ ID NO:19) (Table 7.2A). In contrast, PKB α does not phosphorylate the peptide KKRNKTL SVA (SEQ ID NO:23) (Table 7.2A) or any other peptide that lacks Arg at position n-3. PKB α and p70 S6 kinase, but not MAPKAP-K1, phosphorylate Thr as well as Ser (Table 7.1A) and can phosphorylate peptides lacking any residue at position n+2 ((Leighton et al., 1995) and Table 7.2A), while PKB α and MAPKAP-K1, but not p70 S6 kinase, can tolerate substitution of both the n-1 and n-2 positions of the peptide RPRTSS F (SEQ ID NO:9) with Ala (Table 7.2B). These differences explain why the peptide RPRAAT F (SEQ ID NO:21) is a relatively specific substrate for PKB α .

Replace the paragraph on page 45, lines 19-27, with the following new paragraph:

One of the best peptide substrates for MAPKAP-K1 and p70 S6 kinase (KKRNR T L SVA) (SEQ ID NO:19) was a poor substrate for PKB α (Table 7.2, peptide 2), despite the presence of Arg at positions n-3 and n-5. The presence of Leu at position n-1 and Val at position n+1 are likely to explain the high Km for phosphorylation, because PKB α

prefers a small hydrophilic residue at the former position and a larger hydrophobic residue at the latter position (Tables 7.1 and 7.2).

Replace the paragraph on page 46, lines 5-7, with the following new paragraph:

Monoclonal antibodies recognising the sequence EFMPME (SEQ ID NO:25) (EE) antibodies and the (EQKLISEEDL) (SEQ ID NO:26) c-Myc were purchased from Boehringer (Lewis, UK).

Replace the paragraph on page 46, lines 13-35, with the following new paragraph:

A DNA construct expressing human GSK3B with the EFMPME (SEQ ID NO:25) (EE) epitope tag at the N-terminus was prepared as follows: A standard PCR reaction was carried out using as a template the human GSK3 β cDNA clone in the pBluescript SK+ vector and the oligonucleotides

GCGGAGATCTGCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAAGACC
(SEQ ID NO:27)

and GCGGTCCGGAACATAGTCCAGCACCAG (SEQ ID NO:28) that incorporate a *bgl* II site (underlined) and a *Bspe* I site (double underlined). A three-way ligation was then set up in which the resulting PCR product was subcloned as a *Bgl*III-*Bspe* I fragment together with the C-terminal *Bspe* I-Cla I fragment of GSK3 β into the *Bgl* II-Cla I sites of the pCMV5 vector (Anderson et al., 1989). The construct was verified by DNA sequencing and purified using the Quiagen plasmid Mega kit according to the manufacturer's protocol. The c-Myc GSK3, BA9 construct encodes GSK3 β in which Ser-9 is mutated to Ala and possesses a c-myc epitope tag at the C-terminus and was prepared as described in Sperber et al., 1995. The c-Myc GSK3 β A9 gene was then subcloned into Xba I/EcoRI sites of the pCMV5 eukaryotic expression vector.

Replace the paragraph on page 49, lines 3-30, with the following new paragraph:

Example 10: basic assay for identifying agents which affect the activity of PKB.

A 40 μ l assay mix was prepared containing protein kinase (0.2U/ml) in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μ M PKI, protein kinase substrate (30 μ M), and the indicated concentration of Ro-318220 or GC 109203X (test inhibitors). After incubation on ice for 10 min the reaction was started by the addition of 10 μ l of 50mM magnesium acetate and 0.5 mM [γ ³²P]ATP (100-200 cpm/pmol). For the assay of mixed isoforms of PKC 20 μ M diacylglycerol, 0.5 mM CaCl₂, and 100 μ M phosphatidylserine were also present in the incubations. The assays were carried out for 15 min at 30°C, then terminated and analysed as described (Alessi 1995). One unit of activity was that amount of enzyme that catalysed the phosphorylation of 1nmol of substrate in 1 min. The final concentration of DMSO in easy assay was 1% (by vol). This concentration of DMSO does not inhibit any of these enzymes. Mixed isoforms of PKC were assayed using histone H1 as substrate, while MAPKAP-K1 β and p70 S6 kinase were assayed using the peptide KKRNRRLSVA (SEQ ID NO:19) (Leighton 1995). Protein kinase B was assayed with the peptide GRPRTSSFAEG (SEQ ID NO:1) [9] and MAPKAP-K2 was assayed with the peptide KKLNRRLSVA (SEQ ID NO:57) (Stokoe 1993). p42 MAP kinase was assayed using MBP, and MAPKK-1, and c-Raf1 were assayed as described in Alessi 1995.

Replace the paragraph on page 51, lines 1-22, with the following new paragraph:

General Materials and Methods. Tissue culture reagents, myelin basic protein (MBP), microcystin-LR, and IGF-1 were obtained from Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk (Bagsvaerd, Denmark), phosphate

free Dulbecco's minimal essential medium (DMEM) from ICN (Oxon, UK), Protein G-Sepharose and CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and fluroisothiocyanate-labelled antimouse IgG from goat from Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were raised in sheep against the peptides RPHFPQFSYSASGTA (SEQ ID NO:29) (corresponding to the last 15 residues of rodent PKB α) and MTSALATMRVDYEQIK (SEQ ID NO:30) (corresponding to residues 352 to 367 of human MAPKAP kinase-2) and affinity purified on peptide-CH-Sepharose. Monoclonal HA antibodies were purified from the tissue culture medium of 12CA5 hybridoma and purified by chromatography on Protein G Sepharose. The peptide RPRHFPQFSYSAS (SEQ ID NO:31), corresponding to residues 465-478 of PKB α , was synthesized on an Applied Biosystems 430A peptide synthesizer. cDNA encoding residues 46-400 of human MAPKAP kinase-2 was expressed in E.coli as a glutathione S-transferase fusion protein and activated with p38/RK MAP KINASE by Mr A.Clifton (University of Dundee) as described previously (Ben-Levy et al., 1995). Replace the paragraph on page 51, lines 24-28, with the

following new paragraph:

Monoclonal antibodies recognising the haemagglutinin (HA) epitope sequence YPYDVPDYA (SEQ ID NO:32), Protein G-Sepharose and histone H2B were obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were purified from rabbit skeletal muscle and rat liver respectively.

Replace the paragraph on page 52, lines 34-40, with the following new paragraph:

Assay of immunoprecipitated PKB α and protein

determinations. Three aliquots of each immunoprecipitate (each comprising only 5% of the total immunoprecipitated PKB α) were assayed for PKB α activity towards the peptide

GRPRTSSFAEG (SEQ ID NO:1) as described previously (Cross et al., 1995). One unit of activity was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min. Protein concentrations were determined by the method of Bradford, 1976.

Replace the paragraph on page 55, lines 18-23, with the following new paragraph:

All peptides used to assay PKB α , and TTYADFIASGRTGRRNAIHD (SEQ ID NO:33) (the specific peptide inhibitor of cyclic AMP dependent protein kinase - PKI) were synthesised on an Applied Biosystems 431A peptide synthesizer. Their purity (> 95%) was established by HPLC and electrospray mass spectrometry, and their concentrations were determined by quantitative amino acid analysis.

Replace the paragraph on page 55, line 25 through page 56, line 32, with the following new paragraph:

Preparation and assay of PKB α . The construction of cytomegalovirus vectors (pCMV5) of the human haemagglutinin epitope-tagged wild type (HA-PKB α) was described previously (Alessi et al., 1996). 293 cells grown on 10 cm dishes were transfected with a DNA construct expressing HA-PKB α using a modified calcium phosphate procedure (Alessi et al., 1996). The cells were deprived of serum for 16h prior to lysis and, where indicated, were stimulated for 10 min in the presence of 50 ng/ml IGF-1 to activate PKB α . The cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β -glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μ M Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μ g/ml leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate centrifuged at 4°C for 10 min at 13,000 x g and the supernatant obtained from one 10 cm dish of cells (2-3 mg protein) was incubated for 60 min

on a shaking platform with 20 μ l of protein G-Sepharose coupled to 10 μ g of HA monoclonal antibody. The suspension was centrifuged for 1 min at 13,000 x g, the Protein G-Sepharose-antibody-HA-PKB α complex washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by vol) 2-mercaptoethanol). The PKB α immunoprecipitates were diluted in Buffer B to an activity of 2.0 U/ml towards the Crosstide peptide GRPRTSSFAEG (SEQ ID NO:1) and 0.1 ml aliquots snap frozen in liquid nitrogen and stored at -80°C. No significant loss of PKB α activity occurred upon thawing the PKB α immunoprecipitates or during storage at -80°C for up to 3 months. The standard PKB α assay (50 μ l) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μ M PKI, 0.2 U/ml PKB α , Crosstide (30 μ M), 10 mM magnesium acetate and 0.1 mM [γ^{32} P]ATP (100-200 cpm/pmol). The assays were carried out for 15 min at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described (Alessi et al., 1995). One unit of activity was that amount of enzyme which catalysed the phosphorylation of 1 nmol of Crosstide in 1 min. The phosphorylation of other peptides, histone H2B and MBP were carried out in an identical manner. All the Crosstide activity in HA-PKB α immunoprecipitates is catalysed by PKB α (see Results) and the PKB α concentration in the immunoprecipitates was estimated by densitometric scanning of Coomassie blue-stained polyacrylamide gels, using bovine serum albumin as a standard. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (Bradford et al., 1976). Michaelis constants (Km) and Vmax values were determined from double reciprocal plots of 1/V against 1/S, where V is the initial rate of

phosphorylation, and S is the substrate concentration. The standard errors for all reported kinetic constants were within $\pm 20\%$, and the data is reported as mean values for 3 independent determinations. Fig 16 shows the results relative to those obtained for unstimulated PKB α .

Replace originally filed page 58 with new page 58 attached hereto.

Replace originally filed page 59 with new page 59 attached hereto.

Between pages 64 and 65 as originally filed, insert new SEQUENCE LISTING pages 64-1 to 64-24, attached hereto.

After page 69, add new page 70 with the following Abstract (a new page 70 is attached):

--Abstract of the Disclosure

A method for screening for agents capable of affecting the activity of kinases GSK3 and PKB is disclosed. The method involves assessing the phosphorylation of PKB on two amino acids on the PKB molecule particularly.--

In the Claims:

Amend claims 10 and 13 to read as follows (pursuant to 37 C.F.R. §1.121(c)(1)(ii), a marked-up copy of the amended claims on a separate sheet accompanies this amendment):

10. (Amended) A peptide having or including the amino acid sequence SEQ ID NO:58: Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are any amino acid, and Hyd is a large hydrophobic residue, or a functional equivalent of such a peptide.

13. (Amended) A peptide as claimed in claim 10, having the amino acid sequence SEQ ID NO:1: GRPRTSSFAEG, or a functional equivalent thereof.

REMARKS

The Notice to File Missing Parts indicated that the application was informal for failing to comply with regulations regarding drawings, abstracts, and sequence listings. Each of these points is addressed below.

Drawings

The Notice indicated that substitute drawings were required because the "drawing sheets do not have the appropriate margin(s)". In response, applicants point out that this is a §120 continuation of a U.S. national stage of a PCT application, and therefore the drawings for this national stage application must comply with PCT Rule 11. The drawings as published in the PCT and as provided by the International Bureau have already been checked and were in compliance with PCT Rule 11. Accordingly, the drawings should be acceptable since the Draftsman "may not impose requirements beyond those imposed by the Patent Cooperation Treaty" (see MPEP §1893.03(f)).

In view of the preceding remarks, applicants request that the requirement for corrected drawings be reconsidered and withdrawn.

Abstract

The Notice indicated that the application did not include an abstract. In response, the application has been amended to include an abstract as required by 37 C.F.R. §1.72(b).

Sequence Listing

In response to the Notice and in compliance with the sequence rules, applicants have enclosed the following:

1. A computer readable form (CRF) copy of a Sequence Listing in the form of a 3 1/2" diskette;
2. A paper copy of the Sequence Listing, pages 64-1 to 64-24; and

3. A statement that the content of the paper and computer readable form are the same and include no new matter.

The sequences presented in the SEQUENCE LISTING are as they appeared in the specification as originally filed. The amendments have added SEQ ID NOs to the specification and claims, where appropriate. No new sequences are presented by any of these amendments, and therefore no new matter is presented. Applicants respectfully request that the Sequence Listing be entered in the specification and maintain that the application now complies with the sequence rules.

Please direct any questions regarding this Amendment to applicants' undersigned attorney.

Respectfully submitted,

November 23, 2001
Date

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below.	
11-23-01	<u>Susan J. Braman</u> Susan J. Braman
Date	Attorney Reg. No.: 34,103

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Marked-Up Version of Paragraph(s):

Page 4, lines 8-19:

The present invention also provides a novel peptide comprising the amino acid sequence Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd (SEQ ID NO:4), where Xaa is any amino acid, Yaa and Zaa are any amino acid (preferably not glycine), and Hyd is a large hydrophobic residue such as Phe or Leu, or a functional equivalent thereof. Represented in single letter code, a suitable peptide would be RXRX'X'S/TF/L (SEQ ID NO:4), where X' can be any amino acid, but is preferably not glycine; glycine can in fact be used, but other amino acids are preferred. Typical peptides include GRPRTSSFAEG (SEQ ID NO:1), RPRAATC (SEQ ID NO:2) or functional equivalents thereof. The peptide is a substrate for measuring PKB activity.

Page 11, lines 22-35:

Mono Q chromatography was performed as described (Burgering et al., 1995), except that the buffer also contained 1 mM EGTA, 0.1 mM sodium orthovanadate and 0.5% (w/v) Triton X-100. Two PKB α antibodies were raised in rabbits against the C-terminal peptide FPQFSYSASSTA (SEQ ID NO:3) and bacterially expressed PH domain of PKB α . The C-terminal antibody was affinity purified (Jones et al., 1991). The activity of PKB towards Crosstide is threefold higher than its activity towards histone H2B and 11-fold higher than its activity towards myelin basic protein, the substrates used previously to assay PKB. Other experimental details and units of protein kinase activity are given in Fig 1.

Page 15, lines 5-22:

To identify the insulin-stimulated protein kinase that inhibits GSK3 in the presence of rapamycin and PD 98059, L6 myotubes were incubated with both compounds and stimulated with insulin. The lysates were then

chromatographed on Mono Q and the fractions assayed with "Crosstide" (GRPRTSSFAEG) (SEQ ID NO:1), a peptide corresponding to the sequence in GSK3 surrounding the serine (underlined) phosphorylated by MAPKAP kinase-1 and p70^{S6k} (Ser 21 in GSK3- α (Sutherland et al., 1994) and Ser 9 in GSK3- β (Sutherland et al., 1993)). Three peaks of Crosstide kinase activity were detected, which were absent if insulin stimulation was omitted or if the cells were first preincubated with the PI 3-kinase inhibitor wortmannin (Fig 2a). Wortmannin (Cross et al., 1994 and Welsh et al., 1994), and the structurally unrelated PI 3-kinase inhibitor LY 294002 (ref 19); (Fig 1b), both prevent the inhibition of GSK3 by insulin.

Page 25, lines 11-34:

Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of PKB α causing partial activation. Ser-473 of PKB α [PKBa] lies in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr (SEQ ID NO:11) found to be conserved in a number of protein kinases that participate in signal transduction pathways (Pearson et al. 1995). In order to identify the Ser-473 kinase(s) we therefore chromatographed rabbit skeletal muscle extracts on CM-Sephadex, and assayed the fractions for protein kinases capable of phosphorylating a synthetic peptide corresponding to residues 465 to 478 of PKB α . These studies identified an enzyme eluting at 0.3 M NaCl which phosphorylated the peptide 465-478 at the residue equivalent to Ser-473 of PKB α . The Ser473 kinase co-eluted from CM-Sephadex with MAP kinase-activated protein (MAPKAP) kinase-2, (Stokoe et al., 1992) which is a component of a stress and cytokine-activated MAP kinase cascade (Rouse et al., 1994; Cuenda et al., 1995). The Ser-473 kinase continued to cofractionate with MAPKAP_

kinase-2 through phenyl-Sepharose, heparin-Sepharose, Mono S and Mono Q and was immunoprecipitated quantitatively by an anti-MAPKAP kinase-2 antibody (Gould et al., 1995) demonstrating that MAPKAP kinase-2 was indeed the Ser-473 kinase we had purified.

Page 33, line 23 through page 34, line 8:

Thr-308 is located in subdomain VIII of the kinase catalytic domain, nine residues upstream of the conserved Ala-Pro-Glu motif, the same position as activating phosphorylation sites found in many other protein kinases. However, Ser-473 is located C-terminal to the catalytic domain in the consensus sequence Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr (SEQ ID NO:11) which is present in several protein kinases that participate in growth factor-stimulated kinase cascades, such as p70 S6 kinase, PKC and p90rsk (Pearson et al., 1995). However, it is unlikely that a common protein kinase phosphorylates this motif in every enzyme for the following reasons. Firstly, phosphorylation of the equivalent site in p70 S6 kinase is prevented by the immunosuppressant drug rapamycin (Pearson et al., 1995) which does not prevent the activation of PKB α by insulin (Cross et al., 1995) or it's [is] phosphorylation at Ser-473 (D. Alessi, unpublished work). Secondly, the equivalent residue in protein kinase C is phosphorylated constitutively and not triggered by stimulation with growth factors (Tsutakawa et al., 1995).

Page 34, line 10 through page 35, line 9:

MAPKAP kinase-2 is a component of a protein kinase cascade which becomes activated when cells are stimulated with interleukin-1 or tumour necrosis factor or exposed cellular stresses (Rouse et al., 1994; Cuenda et al., 1995). MAPKAP kinase-2 phosphorylates PKB α

stoichiometrically at Ser-473 (Fig 11) and this finding was useful in establishing the role of Ser473 phosphorylation in regulating PKB α activity. However, although MAPKAP kinase-2 activity is stimulated to a small extent by insulin in L6 cells, no activation could be detected in 293 cells in response to insulin or IGF-1. Moreover, exposure of L6 cells or 293 cells to a chemical stress (0.5 mM sodium arsenite) strongly activated MAPKAP kinase-2 (D. Alessi, unpublished work) as found in other cells (Rouse et al., 1994; Cuenda et al., 1995), but did not activate PKB α at all. Furthermore, the drug SB 203580 which is a specific inhibitor of the protein kinase positioned immediately upstream of MAPKAP kinase-2 (Cuenda et al., 1995), prevented the activation of MAPKAP kinase-2 by arsenite but had no effect on the activation of PKB α by insulin or IGF-1. Finally, the activation of PKB α was prevented by wortmannin (Figs 6 and 9), but wortmannin had no effect on the activation of MAPKAP kinase-2 in L6 or 293 cells. It should also be noted that the sequence surrounding Ser-473 of PKB α (HFPQFSY) (SEQ ID NO:12) does not conform to the optical consensus for phosphorylation by MAPKAP kinase-2 which requires Arg at position n-3 and a bulky hydrophobic residue at position n-5, (where n is the position of the phosphorylated residue). The Km for phosphorylation of the synthetic peptide comprising residues 465-478 is nearly 100-fold higher than the Km for the standard MAPKAP kinase-2 substrate peptide (data not shown). It is therefore unlikely that MAPKAP kinase-2 mediates the phosphorylation of Ser-473 in vivo.

Page 37, lines 10-31:

The activation of PI 3-kinase is essential for many of the effects of insulin and growth factors, including the

stimulation of glucose transport, fatty acid synthesis and DNA synthesis, protection of cells against apoptosis and actin cytoskeletal rearrangements (reviewed in Carpenter et al., 1996). These observations raise the question of whether PKB α mediates any of these events by phosphorylating other proteins. To address this issue we characterised the substrate specificity requirements of PKB α . We find that the optimal consensus sequence for phosphorylation by PKB α is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd (SEQ ID NO:4), where Yaa and Zaa are small amino acids (other than glycine) and Hyd is a large hydrophobic residue (such as Phe or Leu). We also demonstrate that PKB α phosphorylates histone H2B (a substrate frequently used to assay PKB α in vitro) at Ser-36 which lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (SEQ ID NO:4). These studies identified a further PKB α substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe) (SEQ ID NO:6) that, unlike other peptides, is not phosphorylated to a significant extent by either p70 S6 kinase or MAPKAP-K1.

Page 38, line 23 through page 39, line 11:

Identification of the residues in histone H2B

phosphorylated by PKB α . Currently, three substrates are used to assay PKB α activity in different laboratories, histone H2B, MBP and Crosstide. PKB α phosphorylated Crosstide with a Km of 4 μ M and a Vmax of 260 U/mg (Table 7.1 A, peptide 1), histone H2B with a Km of 5 μ M and a Vmax of 68 U/mg, and MBP with a Km of 5 μ M and a Vmax of 25 U/mg. Thus the Vmax of histone H2B and MBP are 4-fold and 10-fold lower than for Crosstide. In order to identify the residue(s) in histone H2B phosphorylated by PKB α , ³²P-labelled histone H2B was digested with trypsin (see Methods) and the resulting peptides chromatographed on a C18 column at pH 1.9. Only one major ³²P-labelled

peptide (termed T1) eluting at 19.5 % acetonitrile was observed (Fig 17A) [,,]. The peptide contained phosphoserine (data not shown), its sequence commenced at residue 34 of histone H2B and a single burst of radioactivity occurred after the third cycle of Edman degradation (Fig 17B), demonstrating that PKB α phosphorylates histone H2B at Ser-36 within the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr (SEQ ID NO:7). Thus, like the serine phosphorylated in Crosstide, Ser-36 of histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (SEQ ID NO:5) (where Hyd is a bulky hydrophobic residue, -Phe in Crosstide, Tyr in H2B).

Page 39, lines 13-32:

Molecular basis for the substrate specificity of PKB α .

To further characterise the substrate specificity requirements for PKB α , we first determined the minimum sequence phosphorylated efficiently by PKB α by removing residues sequentially [sequestly] from the C-terminal and N-terminal end of Crosstide. Removal of the N-terminal glycine and up to three residues from the C-terminus had little effect on the kinetics of phosphorylation by PKB α (Table 7.1A, compare peptides 1 and 5). However any further truncation of either the N or C-terminus virtually abolished phosphorylation (Table 7.1A, peptides 8 and 9). The minimum peptide phosphorylated efficiently by PKB α (Arg-Pro-Arg-Thr-Ser-Ser-Phe) (SEQ ID NO:9) was found to be phosphorylated exclusively at the second serine residue as expected. Consistent with this finding, a peptide in which this serine was changed to alanine was not phosphorylated by PKB α (Table 7.1A, peptide 7). All further studies were therefore carried out using variants of peptide 5 in Table 7.1A (see below).

Page 40, line 29 through page 41, line 8:

Replacement of the Thr situated two residues N-terminal to the phosphorylated serine increased the Km with any amino acid tested (Table 7.1C). Substitution with Ala only increased Km by 2-3 fold, but substitution with other residues was more deleterious and with Asn (a residue of similar size and hydrophilicity to Thr) phosphorylation was almost abolished (Table 7.1C). Replacement of the Ser situated one residue N-terminal to the phosphorylated serine also increased the Km with any amino acid tested, but the effects were less severe than at position n-2 (Table 7.1C). When residues n-2 and n-1 were both changed to Ala, the resulting peptide RPRAASF (SEQ ID NO:13) was phosphorylated by PKB α with a Km only 5-fold higher than RPRTSSF (SEQ ID NO:9). In contrast the peptides RPRGGSF (SEQ ID NO:15), RPRAGSF (SEQ ID NO:16), and RPRGASF (SEQ ID NO:17) were phosphorylated less efficiently (Table 7.1C).

Page 41, lines 21-29:

MAPKAP kinase-1 and p70 S6 kinase phosphorylate the peptides KKKNRTLVA (SEQ ID NO:18) and KKRNRTLVA (SEQ ID NO:19) with extremely low Km values of 0.2- 3.3 μ M, respectively (Table 7.2). However, these peptides were phosphorylated by PKB α with 50-900 fold higher Km values (Table 7.2A, peptides 1 and 2). The peptide KKRNRTLTV (SEQ ID NO:20), which is a relatively specific substrate for p70 S6 kinase (Leighton et al., 1995) was also phosphorylated very poorly by PKB α (Table 7.2A, peptide 4).

Page 41, line 31 through page 42, line 23:

Crosstide is phosphorylated by p70 S6 kinase and MAPKAP kinase-1 with similar efficiency to PKB α ((Leighton et al., 1995); Table 7.2B-peptide 1 and Fig 18). However,

truncation of Crosstide to generate the peptide RPRTSSF (SEQ ID NO:9) was deleterious for phosphorylation by MAPKAP-K1 and even worse for p70 S6 kinase (Table 7.2B-peptides 1 and 2 and Fig 18). Moreover, changing the phosphorylated serine in RPRTSSF (SEQ ID NO:9) to threonine increased the Km for phosphorylation by p70 S6 kinase much more than for PKB α and almost abolished phosphorylation by MAPKAP-K1 (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF (SEQ ID NO:13) was phosphorylated by MAPKAP-K1 with essentially identical kinetics to that of PKB α ; however phosphorylation by p70 S6 kinase was virtually abolished (Table 7.2B-peptide 4 and Fig 18). Based on these observations we synthesized the peptide RPRAATF (SEQ ID NO:21). This peptide was phosphorylated by PKB α with a Km of 25 μ M and similar Vmax to RPRTSSF (SEQ ID NO:9), but was not phosphorylated to a significant extent by either MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig 18). In Fig 18, the protein kinase concentration in the assays towards Crosstide were 0.2 U/ml, and each peptide substrate was assayed at a concentration of 30 μ M. Filled bars denote PKB α activity, hatched bars MAPKAP kinase-1 activity, and grey bars p70 S6 kinase activity. The activities of each protein kinase are given relative to their activity towards Crosstide (100). The results are shown \pm SEM for two experiments each carried out in triplicate.

Page 42, line 25 through page 43, line 3:

Discussion.

We have established that the minimum consensus sequence for efficient phosphorylation by PKB α is Arg-Xaa-Arg-Yaa-Zaa-Ser-Hyd (SEQ ID NO:10), where Xaa is any amino acid, Yaa and Zaa are small amino acids other than glycine (Ser, Thr, Ala) and Hyd is a bulky hydrophobic residue

(Phe, Leu) (Table 7.1). The heptapeptide with the lowest Km value was RPRTSSF (SEQ ID NO:9), its Km of 5 μ M being comparable to many of the best peptide substrates identified for other protein kinases. The Vmax for this peptide (250 nmoles min⁻¹ mg⁻¹) may be an underestimate because the PKB α was obtained by immunoprecipitation from extracts of IGF-1 stimulated 293 cells over-expressing this protein kinase, and a significant proportion of the PKB α may not have been activated by IGF-1 treatment.

Page 43, lines 5-20:

The requirement for arginine residues at positions n-3 and n-5 (where n is the site of phosphorylation) seems important, because substituting either residue with lysine decreases phosphorylation drastically. Serine and threonine residues were preferred at positions n-1 and n-2, although the Km value was only increased about 5-fold if both of these residues were changed to Ala. Serine was preferred at position n, since changing it to threonine caused a six-fold increase in the Km. The peptide RPRAATF (SEQ ID NO:21), which was phosphorylated with a Km of 25 μ M and similar Vmax to RPRTSSF (SEQ ID NO:9), may therefore be a better substrate for assaying PKB α in partially purified preparations, because unlike Crosstide, it contains only one phosphorylatable residue and is not phosphorylated significantly by MAPKAP-K1 or p70 S6 kinase (Table 7.2, Fig 18 and see below).

Page 43, lines 22-35:

The Proline at position n-4 was not altered in this study because it was already clear that this residue was not critical for the specificity of PKB α . Residue n-4 is proline in GSK3 β but alanine in GSK3 α . Both GSK3 isoforms are equally good substrates for PKB α in vitro (Cross et al., 1995), and the peptide GRARTSSFA (SEQ ID

NO:22) (corresponding to the sequence in GSK3 α) is phosphorylated by PKB α with a K_m of 10 μ M and V_{max} of 230 U/mg (Table 7.1A, peptide 2). Moreover, in histone H2B, the residue located four amino acids N-terminal to the PKB α phosphorylation site is serine (Fig 17). The presence of Glu and Lys at positions n-1 and n-2 may explain why histone H2B is phosphorylated by PKB α with a four-fold lower V_{max} than the peptide RPRTSSF (SEQ ID NO:9).

Page 44, line 22 through page 45, line 17:

Additional similarities between p70 S6 kinase, MAPKAP-K1 and PKB α include the failure to phosphorylate peptides containing Pro at position n+1 and dislike of a lysine at the same position. This suggests that, in vivo, these kinases are unlikely to phosphorylate the same residues as MAP kinases (which phosphorylate[s] Ser/Thr-Pro motifs) or protein kinase C (which prefers basic residues C-terminal to the site of phosphorylation). However, the present work has also revealed significant differences in the specificities of these enzymes. In particular MAPKAP-K1 and (to a lesser extent) p70 S6 kinase can tolerate substitution of the Arg at position n-5 by lysine whereas PKB α cannot (see Table 7.1A, Table 7.2A and (Leighton et al., 1995)). MAPKAP-K1 and p70 S6 kinase can also tolerate, to some extent, substitution of Arg at position n-3 by Lys. For example, the peptide KKRNKTL SVA (SEQ ID NO:23) is phosphorylated by MAPKAP-K1 and p70 S6 kinase with K_m values of 17 and 34 μ M, respectively, as compared to K_m values of 0.7 and 1.5 μ M for the peptide KKRNR T L SVA (SEQ ID NO:19) (Table 7.2A). In contrast, PKB α does not phosphorylate the peptide KKRNKTL SVA (SEQ ID NO:23) (Table 7.2A) or any other peptide that lacks Arg at position n-3. PKB α and p70 S6

kinase, but not MAPKAP-K1, phosphorylate Thr as well as Ser (Table 7.1A) and can phosphorylate peptides lacking any residue at position n+2 ((Leighton et al., 1995) and Table 7.2A), while PKB α and MAPKAP-K1, but not p70 S6 kinase, can tolerate substitution of both the n-1 and n-2 positions of the peptide RPRTSSF (SEQ ID NO:9) with Ala (Table 7.2B). These differences explain why the peptide RPRAATF (SEQ ID NO:21) is a relatively specific substrate for PKB α .

Page 45, lines 19-27:

One of the best peptide substrates for MAPKAP-K1 and p70 S6 kinase (KKRNRTLVA) (SEQ ID NO:19) was a poor substrate for PKB α (Table 7.2, peptide 2), despite the presence of Arg at positions n-3 and n-5. The presence of Leu at position n-1 and Val at position n+1 are likely to explain the high K_m for phosphorylation, because PKB α prefers a small hydrophilic residue at the former position and a larger hydrophobic residue at the latter position (Tables 7.1 and 7.2).

Page 46, lines 5-7:

Monoclonal antibodies recognising the sequence EFMPME (SEQ ID NO:25) (EE) antibodies and the (EQKLISEEDL) (SEQ ID NO:26) c-Myc were purchased from Boehringer (Lewis, UK).

Page 46, lines 13-35:

A DNA construct expressing human GSK3B with the EFMPME (SEQ ID NO:25) (EE) epitope tag at the N-terminus was prepared as follows: A standard PCR reaction was carried out using as a template the human GSK3 β cDNA clone in the pBluescript SK+ vector and the oligonucleotides

GCGGAGATCTGCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAAGACC
(SEQ ID NO:27)

and GCGGTCCCGGAACATAGTCCAGCACCAG (SEQ ID NO:28) that incorporate a *bgl* II site (underlined) and a *Bspe* I site (double underlined). A three-way ligation was then set up in which the resulting PCR product was subcloned as a *Bgl*III-*Bspe* I fragment together with the C-terminal *Bspe* I-Cla I fragment of GSK3 β into the *Bgl* II-Cla I sites of the pCMV5 vector (Anderson et al., 1989). The construct was verified by DNA sequencing and purified using the Quiagen plasmid Mega kit according to the manufacturer's protocol. The c-Myc GSK3, BA9 construct encodes GSK3 β in which Ser-9 is mutated to Ala and possesses a c-myc epitope tag at the C-terminus and was prepared as described in Sperber et al., 1995. The c-Myc GSK3 β A9 gene was then subcloned into Xba I/EcoRI [xba I/ECORI] sites of the pCMV5 eukaryotic expression vector.

Page 49, lines 3-30:

Example 10: basic assay for identifying agents which affect the activity of PKB.

A 40 μ l assay mix was prepared containing protein kinase (0.2U/ml) in 50 mM Tris/HCl [Tris/HCl] pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μ M PKI, protein kinase substrate (30 μ M), and the indicated concentration of Ro-318220 or GC 109203X (test inhibitors). After incubation on ice for 10 min the reaction was started by the addition of 10 μ l of 50mM magnesium acetate and 0.5 mM [γ^{32} P]ATP (100-200 cpm/pmol). For the assay of mixed isoforms of PKC 20 μ M diacylglycerol, 0.5 mM CaCl₂, and 100 μ M phosphatidylserine were also present in the incubations. The assays were carried out for 15 min at 30°C, then terminated and analysed as described (Alessi 1995). One unit of activity was that amount of enzyme that catalysed the phosphorylation of 1nmol of [os] substrate in 1 min.

The final concentration of DMSO [DMSO] in easy assay was 1% (by vol). This concentration of DMSO does not inhibit any of these enzymes. Mixed isoforms of PKC were assayed using histone H1 as substrate, while MAPKAP-K1 β and p70 S6 kinase were assayed using the peptide KKRNRTLSVA (SEQ ID NO:19) (Leighton 1995). Protein kinase B was assayed with the peptide GRPRTSSFAEG (SEQ ID NO:1) [9] and MAPKAP-K2 was assayed with the peptide KKLNRRTLSVA (SEQ ID NO:57) (Stokoe 1993). p42 MAP kinase was assayed using MBP, and MAPKK-1, and c-Raf1 were assayed as described in Alessi 1995.

Page 51, lines 1-22:

General Materials and Methods. Tissue culture reagents, myelin basic protein (MBP), microcystin-LR, and IGF-1 were obtained from Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk (Bagsvaerd, Denmark), phosphate free Dulbecco's minimal essential medium (DMEM) from ICN (Oxon, UK) [(ICN, Oxon, UK)], Protein G-Sepharose and CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and fluroisothiocyanate-labelled [fluroisothiocyanate-labelled] antimouse IgG from goat from Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were raised in sheep against the peptides RPHFPQFSYSASGTA (SEQ ID NO:29) (corresponding to the last 15 residues of rodent PKB α) and MTSALATMRVDYEQIK (SEQ ID NO:30) (corresponding to residues 352 to 367 of human MAPKAP_kinase-2) and affinity purified on peptide-CH-Sepharose. Monoclonal HA antibodies were purified from the tissue culture medium of 12CA5 hybridoma and purified by chromatography on Protein G-Sepharose. The peptide RPRHFPQFSYSAS (SEQ ID NO:31), corresponding to residues 465-478 of PKB α , was synthesized on an Applied Biosystems

430A peptide synthesizer. cDNA encoding residues 46-400 of human MAPKAP kinase-2 was expressed in E.coli as a glutathione S-transferase fusion protein and activated with p38/RK MAP KINASE by Mr. A.Clifton (University of Dundee) as described previously (Ben-Levy et al., 1995).

Page 51, lines 24-28:

Monoclonal antibodies recognising the haemagglutinin (HA) epitope sequence YPYDVPDYA (SEQ ID NO:32), Protein G-Sepharose and histone H2B were obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were purified from rabbit skeletal muscle and rat liver respectively.

Page 52, lines 34-40:

Assay of immunoprecipitated PKB α and protein

determinations. Three aliquots of each immunoprecipitate (each comprising only 5% of the total immunoprecipitated PKB α) were assayed for PKB α activity towards the peptide GRPRTSSFAEG (SEQ ID NO:1) as described previously (Cross et al., 1995). One unit of activity was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min. Protein concentrations were determined by the method of Bradford, 1976.

Page 55, lines 18-23:

All peptides used to assay PKB α , and TTYADFIASGRTGRRNAIHD (SEQ ID NO:33) (the specific peptide inhibitor of cyclic AMP dependent protein kinase - PKI) were synthesised on an Applied Biosystems 431A peptide synthesizer. Their purity (> 95%) was established by HPLC and electrospray mass spectrometry, and their concentrations were determined by quantitative amino acid analysis.

Page 55, line 25 through page 56, line 32:

Preparation and assay of PKB α . The construction of cytomegalovirus vectors (pCMV5) of the human

haemagglutinin epitope-tagged wild type [] (HA-PKB α) was described previously (Alessi et al., 1996). 293 cells grown on 10 cm dishes were transfected with a DNA construct expressing HA-PKB α using a modified calcium phosphate procedure (Alessi et al., 1996). The cells were deprived of serum for 16h prior to lysis and, where indicated, were stimulated for 10 min in the presence of 50 ng/ml IGF-1 to activate PKB α . The cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β -glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μ M Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μ g/ml leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate centrifuged at 4°C for 10 min at 13,000 x g and the supernatant obtained from one 10 cm dish of cells (2-3 mg protein) was incubated for 60 min on a shaking platform with 20 μ l of protein G-Sepharose coupled to 10 μ g of HA monoclonal antibody. The suspension was centrifuged for 1 min at 13,000 x g, the Protein G-Sepharose-antibody-HA-PKB α complex washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by vol) 2-mercaptoethanol). The PKB α immunoprecipitates were diluted in Buffer B to an activity of 2.0 U/ml towards the Crosstide peptide GRPRTSSFAEG (SEQ ID NO:1) and 0.1 ml aliquots snap frozen in liquid nitrogen and stored at -80°C. No significant loss of PKB α activity occurred upon thawing the PKB α immunoprecipitates or during storage at -80°C for up to 3 months. The standard PKB α [] assay (50 μ l) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μ M PKI, 0.2 U/ml PKB α , Crosstide (30

μM), 10 mM magnesium acetate and 0.1 mM [$\gamma^{32}\text{P}$]ATP (100-200 cpm/pmol). The assays were carried out for 15 min at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described (Alessi et al., 1995). One unit of activity was that amount of enzyme which catalysed the phosphorylation of 1 nmol of Crosstide in 1 min. The phosphorylation of other peptides, histone H2B and MBP were carried out in an identical manner. All the Crosstide activity in HA-PKB α immunoprecipitates is catalysed by PKB α (see Results) and the PKB α concentration in the immunoprecipitates was estimated by densitometric scanning of Coomassie blue-stained polyacrylamide gels, using bovine serum albumin as a standard. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (Bradford et al., 1976). Michaelis constants (K_m) and V_{max} values were determined from double reciprocal plots of $1/V$ against $1/S$, where V is the initial rate of phosphorylation, and S is the substrate concentration. The standard errors for all reported kinetic constants were within $\pm 20\%$, and the data is reported as mean values for 3 independent determinations. Fig 16 shows the results relative to those obtained for unstimulated PKB α .



S. Serial No. 09/845,667
Marked-Up Version of Claim(s):

10. (Amended) A peptide having or including the amino acid sequence SEQ ID NO:58: Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are any amino acid, and Hyd is a large hydrophobic residue, or a functional equivalent of such a peptide.

13. (Amended) A peptide as claimed in claim 10, having the amino acid sequence SEQ ID NO:1: GRPRTSSFAEG, or a functional equivalent thereof.

Table 7.1

Molecular basis for the substrate specificity of PKB α

The phosphorylated residue is shown in boldface type, the altered residue is underlined>. V(100 μ M) is the relative rate of phosphorylation at 0.1 mM peptide relative to peptide 1. ND, not determined. *An alanine residue was added to the C-terminal of the peptide RPRTSSP, since we have experienced difficulty in synthesising peptides terminating in proline.

A	Peptides	Seq ID No	Km (μ M)	Vmax (U/mg)	V(0.1 mM)
1.	GRPRTSSF AEG	1	4	250	100
2.	RPRTSSFA	34	8	305	109
3.	GRPRTSSF	35	8	385	129
4.	RPRTSSF	9	5	260	105
5.	RPRTSTF	36	30	243	78
6.	RPRTSAF	37	--	0	
7.	PRTSSF	38	--	0	
8.	RPRTSS	39	> 500	ND	2
9.	KPRTSSF	40	> 500	ND	4
10.	RPKTSSF	41	> 500	ND	2
B					
1.	RPRTSSF	9	5	260	105
2.	RPRTSSL	42	8	278	104
3.	RPRTSSV	43	21	300	102
4.	RPRTSSA	44	250	265	30
5.	RPRTSSK	45	80	308	67
6.	RPRTSSE	46	> 500	ND	9
7.	RPRTSSPA*	47	--	0	
C					
1.	RPRTSSF	9	5	260	105
2.	RPRASSF	48	12	230	89
3.	RPRVSSF	49	25	273	77
4.	RPRGSSF	50	60	163	37
5.	RPRNSSF	51	> 500	ND	21
6.	RPRTASF	52	20	213	83
7.	RPRTGSF	53	25	233	77
8.	RPRTVSF	54	30	365	89
9.	RPRTNSF	55	30	300	81
10.	RPRAASF	13	25	215	77
11.	RPRGGSF	15	105	345	55
12.	RPRGASF	17	105	160	37
13.	RPRAGSF	16	49	114	70

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Table 7.2 Comparison of the substrate specificities of PKB α , MAPKAP kinase-1, and P70S6 kinase. Peptides 1 and 2 are very good substrates for MAPKAP kinase-1 and p70 S6 kinase, and peptide 3 is a relatively specific substrate for p70 S6 kinase [16]. *Data reported previously [16]; ND, not determined.

A	Peptide	Seq ID No	K _m (mM)	Protein kinase B α	K _m (mM)	MAPKAP kinase-1	K _m (mM)	V _{max} (U/mg)	p70 S6 kinase
1.	KKKNRTLVA	18	185	270	0.2*	V _{max} (U/mg)	K _m (mM)	V _{max} (U/mg)	
2.	KKRNRTLVA	19	80	300	0.7*	1550*	3.3*	890*	
3.	KKRNKTLVA	23	>500	ND	17*	1800*	1.5*	1520*	
4.	KKRNRTLTV	20	388	330	40*	840*	34*	760*	
B						270*	4.8*	1470*	
1.	GRPTSSFAEG	1	4	250	2	790	3	1270	
2.	RPRTSSF	9	5	260	12	840	125	705	
3.	RPRTSTF	36	30	240	>500	ND	211	590	
4.	RPRAASF	13	25	215	20	1020	>500	ND	
5.	RPRAATF	21	25	230	>500	ND	>500	ND	